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In vitro and in vivo evaluation of effects of sodium caprate on enteral peptide absorption and on mucosal morphology

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Abstract

Sodium salts of medium-chain fatty acids, sodium caprate (C10) in particular, have been used as absorption-enhancing agents to promote transmucosal drug absorption. In this study, we conducted both in vitro and in vivo experiments to investigate the effects of C10 on intestinal permeabilities and mucosal morphology. Mucosal addition of C10 (13–25 mM) reduced the transepithelial electric resistance (TEER) of cultured monolayers of the human intestinal cell line Caco-2 by 40–65% and, upon removal of C10, a marked tendency of TEER recovery was recorded. C10 added mucosally at 13–50 mM increased the transports of mannitol and polyethylene glycol (PEG) 900 across Caco-2 in a dose-dependent manner. In contrast, the transport of a model D-decapeptide was maximally enhanced with 20–25 mM C10. No noticeable morphological alteration of the Caco-2 monolayers was observed after a 1-h mucosal pretreatment with C10. Co-delivery with C10 (0.05–0.5 mmol/kg) into the rat terminal ileum increased the D-decapeptide bioavailability (BA) dose-dependently. With 0.5 mmol/kg C10 co-administered, D-decapeptide percent BA was elevated from 2 to 11%. Following a 1-h incubation with 0.5 mmol/kg C10 (in liquid or powder form) non-invasively delivered into the rectal lumen, no signs of histological change in the rectal mucosa were detected. These results demonstrate that C10 can promote intestinal absorption of a small peptide without causing detrimental alterations of the intestinal mucosa. C10 thus seems to be a good candidate as an enhancing agent for improving the oral BA of small therapeutic peptides.

Keywords: Absorption-enhancing agent; Caco-2; Intestinal transport; Model peptide; Mucosal morphology; Sodium caprate

Abbreviations: BA, bioavailability; C10, sodium caprate; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; PEG, polyethylene glycol; TEER, transepithelial electric resistance; V_E, transepithelial voltage difference.

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1. Introduction

Application of absorption-enhancing agents as formulation excipients to improve the oral bioavailability (BA) of biopharmaceuticals has remained an active area of research in drug delivery technology (Lee et al., 1991; Aungst, 1996). In recent years, particular attention has been given to enhancing agents that are endogenous and/or metabolizable by mucosal tissues (Fix, 1987; Muranishi, 1990; Chao et al., 1998a). As a good example, sodium salts of medium-chain fatty acids, sodium caprate (C10) in particular, have been shown to promote the transmucosal absorption of drugs and marker compounds (Tomita et al., 1992; Lindmark et al., 1998; Shimazaki et al., 1998), apparently mainly by enhancing the permeabilities of the paracellular pathway (Sawada et al., 1991; Anderberg et al., 1993). C10 is a dairy product constituent that has been approved in a few countries for use in humans as an absorption-enhancing agent (Watts and Illum, 1997).

It seems that so far most of the published studies on effects of C10 on intestinal permeabilities have involved in vitro transport analysis of small-molecule, non-peptide marker compounds. Also, data are sparse regarding the in vitro and in vivo effect of C10 on morphology/viability of the mucosal epithelial tissues. To complement the information available in the literature, in the present study we conducted both in vitro and in vivo investigations of effects of C10 on intestinal absorption of model compounds including a peptidase-resistant model decapeptide, and on mucosal morphology. Our results show that C10, when co-administered as a formulation excipient, can effectively enhance the intestinal absorption of small peptide and other small-molecule compounds without causing significant morphological alterations of the gastrointestinal (GI) mucosa.

2. Materials and methods

2.1. Materials

Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). Male Sprague–Dawley rats were from Charles River (Wilmington, MD). Cell culture media and reagents were from Life Technologies (Grand Island, NY). Fetal bovine serum was from HyClone Laboratories (Logan, UT). Synthetic D-decapeptide (constituent amino acid sequence: D-Glu-D-Tyr-D-Leu-D-Lys-D-Ala-D-Leu-D-Leu-D-Ser-D-Lys-D-Leu-NH_2) and D-[^{3}H]decapeptide (35 Ci/mmol) were from Cambridge Research Biochemicals (Wilmington, DE). C10, mannitol, and formalin solution (10%) were from Sigma (St Louis, MO). Polyethylene glycol (average molecular weight, 900 Da; PEG900) was from Aldrich (Milwaukee, WI). [^{14}C]Mannitol (55 mCi/mmol) and [^{3}H]PEG900 (6.75 mCi/mmol) were from American Radiolabeled Chemicals (St Louis, MO). EcoLite liquid scintillation fluid was from E&K Scientific Products (Saratoga, CA).

2.2. Cell culture

Caco-2 cells (passage 23–41) were maintained in high-glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, nonessential amino acids, penicillin, and streptomycin. Cells were plated on rat-tail collagen-precoated 12-mm Snapwell (pore size 0.4 μm) or Transwell (pore size 3 μm) polycarbonate membrane inserts (surface area 1 cm^2; Costar, Cambridge, MA) at a density of 6×10^4 cells/cm^2. Confluent cell monolayers were studied ~ 3 weeks following seeding.

2.3. Transepithelial electrophysiology

The transepithelial electric resistance (TEER) of cultured monolayers of Caco-2 cells was measured as described elsewhere (Chao et al., 1998a,b). Briefly, the cell monolayers grown on the Snapwell membrane supports were mounted vertically in a side-by-side acrylic perfusion chamber system (Costar) and bathed on both sides with freshly prepared Kreb's buffer (pH 7.4), which was continuously oxygenated and mechanically stirred by a 95:5 O_2/CO_2 (%) gas-lift system at 37°C. A pair of voltage- and current-sensing electrodes connected to a voltage-clamp amplifier (Physiologic Instruments, San Diego, CA) was inserted into

the appropriate half-chambers across the cell monolayer. The transepithelial potential difference (V_E) was clamped to zero, and the short-circuit current was recorded with the aid of ACQUIRE & ANALYZE data acquisition software (Physiologic Instruments) on a PC computer and displayed on a monitor. TEER was determined from the magnitude of the 250-ms current pulses passed across the short-circuited cell monolayer, at 20-s intervals, to displace the V_E by 1 mV from zero, according to Ohm's law (Chao and Mochizuki, 1992; Chao et al., 1998b). Only the cell monolayers with a baseline TEER of ≥ 200 Ω-cm^2 were studied. C10 was pipetted into the mucosal half-chamber from a concentrated stock solution. To examine the reversibility of the effect of C10, 15 min after the mucosal addition of C10, the mucosal bathing solution was replaced with fresh 95:5 O_2/CO_2 (%) pregassed Kreb's solution using peristaltic tubing pumps (VWR Scientific Products, San Francisco, CA), and the TEER of the Caco-2 monolayers was continuously recorded. The TEER values measured from each cell monolayer were normalized as a percentage of the baseline TEER value recorded prior to addition of C10.

2.4. Transepithelial flux experiments

To examine the effect of C10 on the mucosal-to-serosal flux of a selected compound, Caco-2 monolayers grown on 12-mm Transwell membrane inserts were first rinsed with Hank's balanced salt solution (HBSS) containing 25 mM glucose and 10 mM HEPES (modified HBSS) and bathed with modified HBSS for ~ 15 min. Then the mucosal (top) bathing solution (0.5 ml) was replaced with modified HBSS containing an appropriate amount of the radioactively labeled compound (0.25–0.5 μCi) plus 0.1 mM of the corresponding unlabeled compound, in the absence (control) or presence of C10 at desired concentrations. The transport rate of the compound in question was examined by sampling the total serosal (bottom) bathing medium (1 ml) at desired time intervals for up to 90 min at room temperature. Samples collected from the serosal (receiver) compartment were mixed with the Eco-Lite liquid scintillation cocktail and counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA).

2.5. In vivo absorption experiments

The protocol for the animal study had been previously approved by the ALZA Institutional Animal Care and Use Committee (IACUC). Fasted Sprague–Dawley rats (300–500 g) were first given an intraperitoneal injection of sodium pentobarbital (40–50 mg/kg body wt). The right jugular vein was catheterized for blood sampling and for administration of additional doses of sodium pentobarbital as needed to maintain an adequate anesthetic plane. The abdominal cavity was opened by a midline incision, and a segment of the terminal ileum was located. Physiological phosphate-buffered saline (PBS; 1 ml/kg) containing 1–1.5 μCi/ml of D-[^{3}H]decapeptide and 2 mg/ml of unlabeled decapeptide, with C10 at desired doses (in the range of 0.05–0.5 mmol) or without C10 (control), was injected into the lumen of the terminal ileum using a 1-ml syringe connected to a 30-gauge needle. Thus D-decapeptide (^{3}H-labeled at specific activity of 0.5–0.75 μCi/mg) was dosed at 2 mg/kg; and when C10 was studied, the PBS-based formulation contained C10 in the concentration range of 0.1–1.67 M. Intravenous (i.v.) injection of the D-decapeptide solution was given via the femoral vein. Blood samples were taken at selected time points for up to 6 h. At the end of the experiment, the rats were euthanized with an i.v. overdose of sodium pentobarbital. The blood samples collected were centrifuged at 9000–10 000 $\times$ g at 4°C for 5 min. The plasma phase of the samples was extracted and then individually mixed with EcoLite liquid scintillation cocktail and counted in a Beckman liquid scintillation counter. Decapeptide absorption was determined by the area under the plasma concentration–time curve (AUC) and its percent bioavailability (%BA). The AUC was determined using the trapezoidal rule; %BA was the percentage of the AUC resulting from intestinal delivery over the normalized AUC following i.v. injection.

2.6. Histological evaluation

To examine the effects of C10 on morphology of Caco-2 cell cultures, the cell monolayers were mucosally pretreated with C10 of desired concentration or with modified HBSS only (control) for 1 h. The bathing solutions were subsequently aspirated and the cell monolayers fixed with 10% formalin for ~48 h. The samples were then sent to Pathology Associates International (Frederick, MD) for histological processing (paraffin embedding, standard H&E staining, sectioning). Photographs were taken from slides of processed samples on a Zeiss Axioskop light microscope (Carl Zeiss, Thornwood, NY) with a Plan-Neofluar 40× oil-immersion objective using a Zeiss 35-mm camera.

The effect of C10 on the GI mucosal morphology was also examined in vivo following direct delivery of C10 to the rectal lumen. The experimental protocol had been previously approved by the ALZA IACUC. Fasted Sprague–Dawley rats were first given an anesthetic induction of 3–5% isoflurane (mixed with 95% oxygen) with a Vasco vaporizer (Omni Medical Equipment, CA) at a flow rate of 2–4 l/min in an anesthesia induction chamber and subsequently maintained on isoflurane with a facemask. A test formulation containing a high dose (0.5 mmol, or 97.1 mg, per kg) of C10 dissolved in PBS at 1 ml/kg (liquid form) or the same dose of C10 powder, or PBS alone (control) was delivered into the rat rectal cavity using a 1-ml syringe attached to a segment of medical-grade polyethylene tubing (i.d. 1–1.5 mm) with flared tip. The test formulation was left within the rectal lumen for incubation of the mucosal surfaces for 1 or 4 h. At the end of the experiment, the rats were sacrificed with an induction of isoflurane followed by an intracardiac injection of sodium pentobarbital overdose. The rectum was removed, opened and pinned down on a dissecting pan, and the tissue samples of the treated rectal mucosa were taken using a 6- to 9-mm biopsy punch. All the mucosal samples were fixed in 10% formalin for at least 48 h. The samples were then sent to Consolidated Veterinary Diagnostics (Sacramento, CA) for histological processing (paraffin embedding, standard H&E staining, sectioning). Photographs were taken from slides of processed samples on a Leica LaborLux 12Pol S light microscope (Leica, Deerfield, IL) with a NPL Fluotar 20× objective, using a CCD video camera system (model LX-450; Optronics Engineering, Goleta, CA). Video images of the mucosal specimen were displayed on a monitor screen and printed on a Mavigraph color video printer (Sony, Japan).

3. Results

3.1. Effect of C10 on the TEER of Caco-2 cell monolayers

The effect of C10 on the in vitro permeability of intestinal epithelium and the reversibility of this effect were first examined electrophysiologically by monitoring the TEER values of cultures of Caco-2 cells. The baseline TEER of the Caco-2 monolayers was 244 ± 8 (S.E.) Ω-cm^2 ($n = 28$). As shown in Fig. 1, the mucosal addition of C10 in the concentration range of 13–25 mM quickly reduced the Caco-2 TEER by 40–65%, with a $T_{1/2}$ (the time required to show the half-maximal effect of C10) of ~5 min. Upon wash-out of C10 from the mucosal half-chamber, a clear trend of recov-

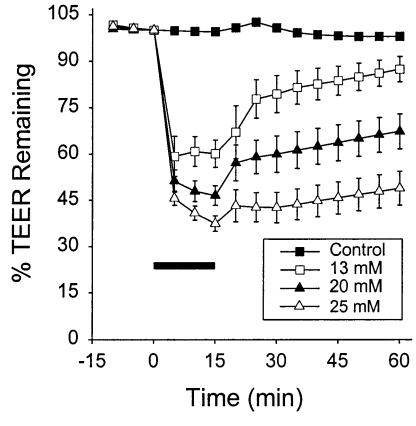

Fig. 1. Effect of mucosal addition of C10 on the time course of TEER of cultured monolayers of Caco-2 cells. C10 was added where indicated by the horizontal bar and removed thereafter. Data shown represent mean ± S.E. of values recorded in at least four cell monolayers.

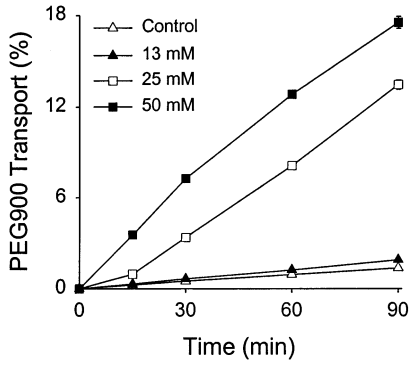

Fig. 2. Effect of mucosal addition of C10 on the time course of cumulative percent transport of PEG900 ($n = 4$ in each condition).

ery of TEER was recorded, suggesting that the permeability-enhancing effect of C10 on the Caco-2 cells is readily reversible.

3.2. Effect of C10 on in vitro transport of marker compounds and D-decapeptide across Caco-2

The permeation-enhancing activity of C10 was further investigated by examining its effect on the transport of small-molecule marker compounds (PEG 900 and mannitol), and a synthetic model D-decapeptide (molecular weight ~1.2 kDa; Chao et al., 1998b) across Caco-2. Fig. 2 shows the effect of C10 on the time course of Caco-2 transport of PEG900 in these in vitro flux experiments. C10 was added only to the mucosal compartment to mimic the condition in which this agent would likely be used as a formulation excipient for in vivo transmucosal drug delivery. The cumulative PEG900 transport across Caco-2 increased slightly with 13 mM C10, but increased at least 10-fold at concentrations of 25 mM and greater.

Fig. 3 summarizes the effect of C10 on the transport of mannitol, PEG900, and D-decapeptide. C10 at concentrations of 20–50 mM elevated mannitol transport dose-dependently with a more pronounced enhancement profile compared with its effect on PEG900 permeation. In contrast, C10 maximally enhanced D-decapeptide permeation at 20–25 mM. The submaximal rate of decapeptide transport in the presence of 50 mM C10 might be due to complexation between decapeptide and C10 in the donor (mucosal) solution (see Section 4).

3.3. Effect of C10 pretreatment on morphology of Caco-2 cell monolayers

The effect of C10 on the morphology of cultures of Caco-2 cells was assessed by histological examination following a 1-h mucosal pretreatment with C10. Again, only the mucosal surfaces of the cell monolayer were exposed to C10, to mimic the condition in which this agent would likely be used as a formulation excipient for in vivo drug delivery. No discernible change in morphology of the cell monolayers was observed after pretreatment with 13 mM C10, albeit some slight morphological alteration (e.g. darkened staining of the cells) was seen following an incubation with 20 mM C10 (Fig. 4). However, after being pretreated with 50 mM for 1 h, the cell monolayer became largely detached from the underlying membrane support on which the cells were cultured or appeared disrupted.

3.4. Effect of co-administration of C10 on the in vivo intestinal absorption of D-decapeptide

The enhancing activity of C10 was further evaluated in vivo to confirm the C10-promoted decapeptide transport shown in Caco-2. Liquid

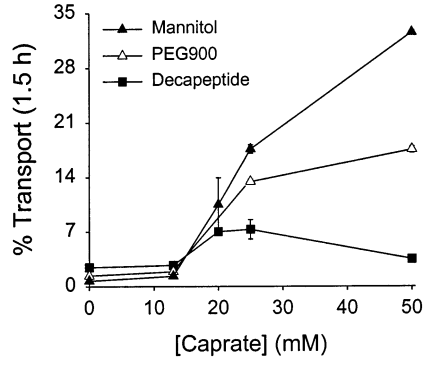

Fig. 3. Effect of mucosal addition of different concentrations of C10 on cumulative percent transport of mannitol, PEG900, and model decapeptide over 1.5 h across Caco-2 cell monolayers ($n = 3$–6 each).

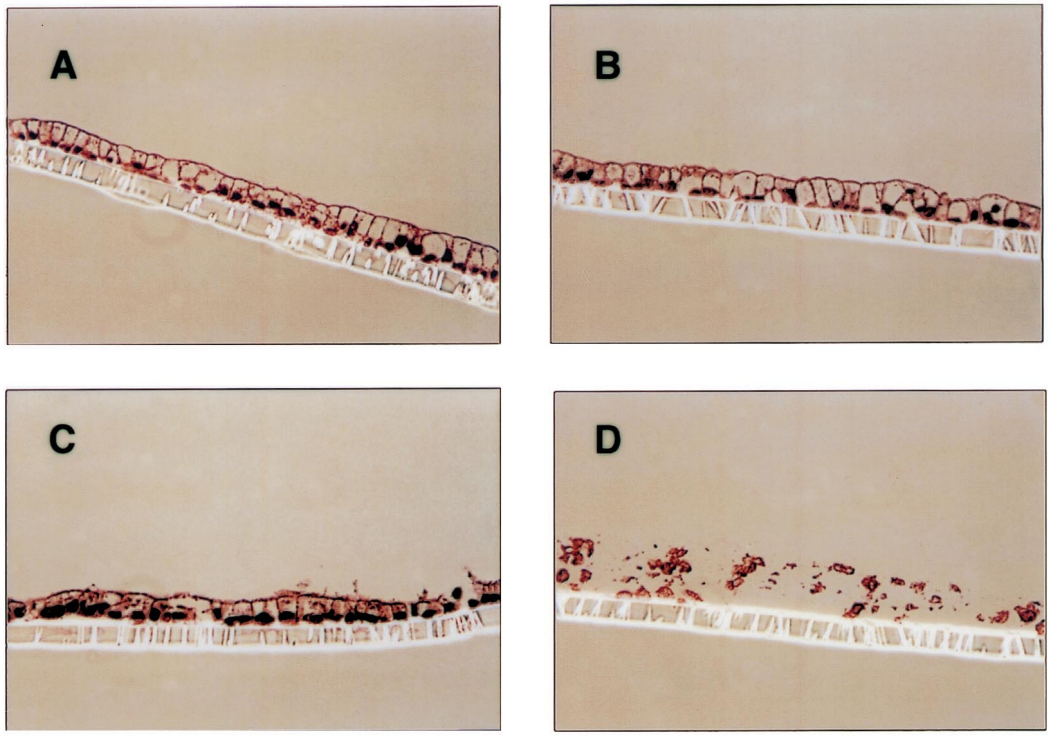

Fig. 4. Histological evaluation of the effect of C10 on the morphology of cultured monolayers of Caco-2 cells. A, control ($n = 5$). B to D, mucosally pretreated with 13 mM ($n = 3$), 20 mM ($n = 3$), and 50 mM ($n = 2$) C10, respectively, for 1 h.

formulation containing D-decapeptide and selected doses of C10 was directly delivered to the lumen of the rat terminal ileum. As shown in Fig. 5, the ileal co-delivery of C10 enhanced the decapeptide percent BA in a dose-dependent fashion. With C10 co-administered at 0.5 mmol/kg, the decapeptide percent BA was increased about fivefold. No noticeable sign of mucosal irritation/damage was observed at the end of the experiments.

3.5. Effect of in vivo luminal incubation of C10 on morphology of rectal mucosa

The effect of C10 on mucosal morphology was also investigated in vivo. The rectal cavity was chosen as the site of testing because of its ready access for non-invasive delivery of formulation. C10 was administered to the rectal lumen at 0.5 mmol (or 97.1 mg) per kg (the highest dose used in the decapeptide BA study), in liquid (pre-dissolved in PBS at 1 ml/kg) or powder form, to pre-incubate the rectal mucosa for 1 or 4 h. As

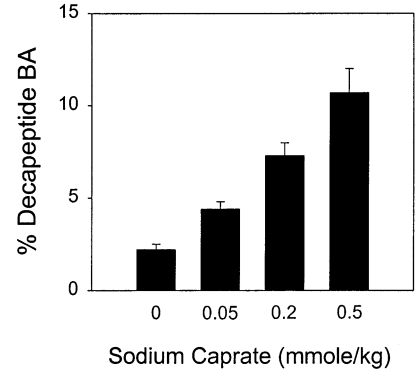

Fig. 5. Effect of ileal co-administration of C10 with model decapeptide on percent decapeptide BA (mean ± S.E.; $n = 3$–7 animals in each condition).

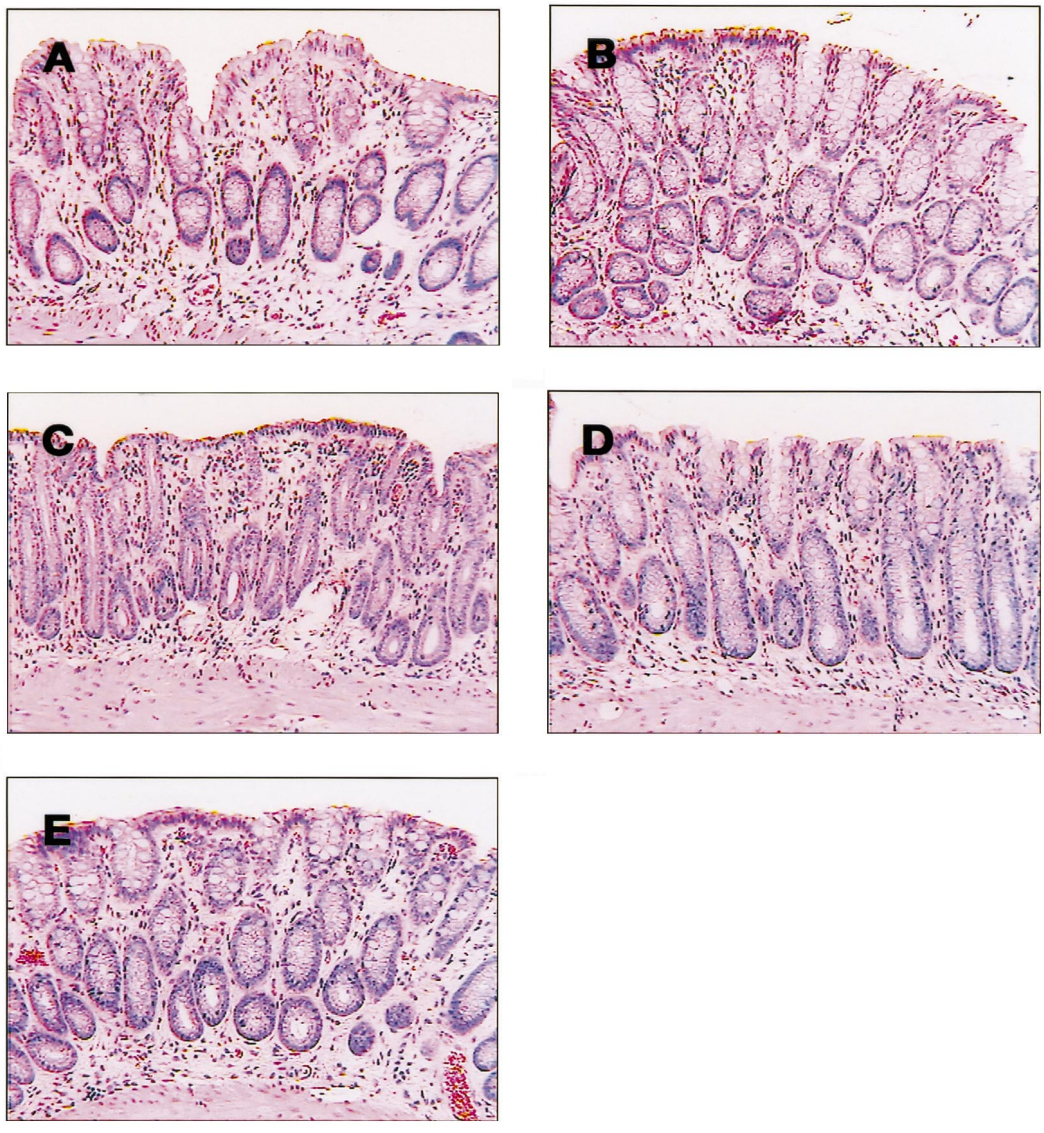

Fig. 6. In vivo histological evaluation of the effect of C10 (dosed at 0.5 mmol, or 97.1 mg, per kg) on morphology of rectal mucosa. A, control. B and C, luminal incubation with C10 (liquid form) for 1 and 4 h, respectively. D and E, luminal incubation with C10 (powder form) for 1 and 4 h, respectively; $n = 4$ animals in each condition.

shown in Fig. 6, no discernible morphological change of the rectal mucosa was observed following the C10 pretreatment compared with the control specimens (incubated with PBS only). This result suggests that C10 is compatible with GI mucosal tissues.

4. Discussion

Mucosal addition of C10 rapidly reduced the Caco-2 TEER ($T_{1/2}$, ~ 5 min) and, upon its washout, a marked propensity for recovery of TEER was observed. This result is in concert with

the recent finding by Söderholm et al. (1998) that apical C10 causes a reversible drop in V_E and increases in vitro EDTA permeation across rat ileum, and suggests that C10 does not cause long-term damage to the Caco-2 monolayer and that its effect on Caco-2 permeability is reversible.

To study the effect of C10 on transepithelial transport, among the test compounds used, we included a synthetic model decapeptide composed of D-amino acids. This D-decapeptide normally forms an α-helix, a common structure for naturally-occurring and therapeutic peptides. D-Oligopeptide tends to be metabolically more stable (would not be recognized by the intestinal peptidases) and thus has been used to characterize intestinal peptide transport (Pappenheimer et al., 1994). The dose-dependent enhancement by C10 of small-molecule hydrophilic marker compound (mannitol and PEG900) permeation across Caco-2 is consistent with the data reported in the literature. Interestingly however, the transport of model decapeptide was maximally enhanced by C10 at 20–25 mM but not at the highest concentration tested (50 mM). The reason for the submaximal transport of model decapeptide in the presence of 50 mM C10 is not clearly understood, but may be due in part to electrostatic interaction between the two compounds. At physiological pH (7.4), the model decapeptide (isoelectric point 9.8) carries one (or two) net positive charge. At concentrations above its critical micelle concentration (13 mM), C10 spontaneously forms micelles with its carboxylate group facing outwards. One possibility is that, in the presence of C10 at a concentration as high as 50 mM, a substantial fraction of decapeptide is attracted onto the surface of and/or entrapped in the micelles formed with C10, resulting in a significantly reduced 'effective' concentration of decapeptide in the mucosal (donor) solution. Some surfactants like Cremophor-EL have been shown to enhance intestinal drug absorption by inhibiting the P-glycoprotein efflux system. However, the accumulated data indicate that C10 increases the intestinal permeability primarily by opening up a paracellular pathway (Sawada et al., 1991; Anderberg et al., 1993). Since the decapeptide is highly hydrophilic, its enhanced transport induced by C10 is most likely to occur via a paracellular route.

The result of the in vitro histological evaluation that lengthy (1 h) incubation of the mucosal surfaces of the Caco-2 culture with 20 mM C10 (which led to maximally enhanced decapeptide permeation) failed to cause any marked change in the cell monolayer morphology appears encouraging, as it is usually anticipated to take much less than 1 h for a formulation agent(s) delivered into the intestinal lumen to become largely diluted by the luminal content. This result is also consistent with the conclusion by Anderberg and colleagues (1993) that C10 enhances Caco-2 permeability mainly by opening up a paracellular route, instead of by causing detrimental damages to the intestinal epithelial cells.

It has been known that the intestinal permeability enhancement brought about in vitro by enhancers may not be replicable in vivo (Scott-Moncrieff et al., 1994). This is because the enhancing agent delivered into the GI tract tends to be diluted quickly by the luminal content, resulting in reduced efficacy. In this study, the enhancing effect of C10 on in vitro intestinal absorption (Caco-2) of model decapeptide was confirmed in vivo; with C10 co-delivered to the terminal ileum, the decapeptide percent BA was enhanced about fivefold. The liquid formulation (PBS-based) containing C10 is fairly viscous, which might be helpful in lessening the dilution effect of the luminal content.

It was also noted that the enhanced decapeptide BA was more markedly dependent on the dose of C10 co-administered compared with the decapeptide transport data obtained in Caco-2 cells. The reason for this apparent difference is not clearly defined. The stability of the micelles formed with C10 however might be affected by the environment in the intestinal lumen; also, the interaction between the D-decapeptide and C10 might be interfered with by the peptides, proteins, and/or fatty acids present. Among the other possibly relevant thermodynamic parameters, it has been shown (Pedersen et al., 1990) that the strength of ionic binding between medium-chain fatty acids and albumin decreases with increasing temperature (in the range of 5–37°C). If this is also true for the ionic interaction between D-decapeptide and C10, the D-decapeptide would be more

strongly bound to the C10-formed micelles during the in vitro flux experiments, which were performed at room temperature.

In the in vitro evaluation, C10 did not seem to cause marked morphological alteration of the Caco-2 monolayer at 13 and 20 mM. However, the monolayer structure appeared disrupted after a 1-h incubation with 50 mM C10. In the in vivo absorption experiments, even when the liquid formulation delivered to the rat ileal lumen (at 1 ml/kg) contained C10 at high concentrations, no perceptible sign of mucosal irritation or damage was seen at the end of those experiments. Conceivably, the C10 delivered to the intestinal lumen was diluted during the course of the experiment to concentrations tolerable by the mucosal tissues. Another possibility is that local, transient mucosal irritation/damage might have been induced initially, but was able to recover over time as the C10 delivered became gradually diluted. The reversibility of the C10 effect on intestinal permeabilities has been demonstrated in both in vitro (Anderberg et al., 1993; this study) and in vivo (Söderholm et al., 1998) experiments. Caco-2 cell monolayers in general seem to be more sensitive to the effects of surfactant permeation-enhancing agents than the whole intestinal tissues (Anderberg and Artursson, 1993). This may be partly because the Caco-2 cell line does not produce a protective mucous layer, the apical cell membranes thus are freely accessible by the enhancing agents administered.

In this study we have also examined more extensively the in vivo effect of C10 on the mucosal morphology in the rectal lumen. The rectal mucosa is readily accessible non-invasively for in vivo delivery of the test formulation and has been used previously for morphological evaluation of the effect of enhancer on the mucosal epithelial cells (Sithigorngul et al., 1983). We failed to detect any discernible morphological alteration in the rat rectal mucosa even 4 h after the delivery of C10 in powder form. Lindmark et al. (1997) reported that rectal delivery of an ampicillin (AM)-C10 formulation resulted in reversible histological mucosal damage. However, they also found that AM plays a role in prolonging mucosal damage and that C10 has a protective effect on the rectal mucosa. Taken together, C10 seems to be fairly compatible with the mucosal tissue.

In conclusion, C10 is effective in promoting the intestinal absorption of small-molecule compounds, including a model decapeptide, without causing significant damage to the intestinal mucosa. C10 thus seems to be a good candidate for a formulation excipient to enhance the oral BA of small therapeutic peptides.

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